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Light and electron microscopic studies of *Averrhoa* (Oxalidaceae) pollen

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Summary. The mature pollen grains of *Averrhoa carambola* L. ("pin-eyed" clone B2 and "thrum-eyed" clone B17) and *A. bilimbi* L. were studied. They are small to medium-sized, oblate spheroidal to subprolate and tricolpate with the colpus broadening at the centre and tapering towards the poles. The tectum ornamentation of the pollen wall is tectate-perforate in both clones of *A. carambola* and semitectate in *A. bilimbi,* while its surface is foveolate in both clones of *A. carambola* and reticulate in *A. bilimbi*. The exine consists of tectum, columellae, foot-layer and endexine. *A. carambola*, clone B17, has a thin and continuous tectum, prominent columellae and a thick foot-layer. *A. bilimbi* has a thick and discontinuous tectum, discrete entities of columellae and a thin foot-layer. The cytoplasm of the pollen grains is occupied by many plastids containing starch.

The genus *Averrhoa* consists of only two species, *viz., A. bilimbi* L. (*belimbing buluh* in Malay) and *A. carambola* L. (carambola or starfruit). Both species are members of the Oxalidaceae and common fruit tree species cultivated in the tropics, particularly in South East Asia (Allen 1967; Veldkamp 1971) where they are indigenous (Samson 1991). The botany and cultivation of these species have been described by Popenoe (1920), Ochse & van der Brink (1931) and Burkill (1935).

The *Averrhoa* tree is monoecious and bears bisexual flowers with ten stamens which are arranged in two whorls. There are two forms of flowers in *A. carambola*: one with long styles (pin-eyed form, clone B2) and the other with short styles (thrum-eyed form, clone

B17); in both forms five of the stamens are staminodes or modified nectaries, short and rudimentary, whereas the other five stamens are functional, with normal anthers. In *A. bilimbi*, on the other hand, all the ten stamens have normal anthers.

There are very few studies of the pollen morphology of the Oxalidaceae. Besides the work of Erdtman (1966) on 11 species from six genera of the Oxalidaceae (including *A. bilimbi*), Low (1972), Tan (1977) and Ou Yang (1978) have studied the pollen morphology of both *Averrhoa* species using the Light Microscope (LM), and Chin & Phoon (1982) have conducted a Scanning Electron Microscope (SEM) study of carambola pollen. In this present study, the pollen morphology of *Averrhoa* was studied using LM, SEM and Transmission Electron Microscope (TEM).

This paper sets out to examine and describe the pollen of the species of *Averrhoa* and ascertain if there are differences in the pollen between the species and whether the pollen varies between pin and thrum flowers.

Materials and methods

Pollen together with anther material from freshly open flowers were obtained from the farm of the Institute for Advanced Studies, University of Malaya (*A. carambola*, clone B2 and clone B17), and the Rimba Ilmu, University of Malaya (*A. bilimbi*) on the 16th August 1991.

For LM studies, the pollen material was dried at 40°C, before being acetolysed, stained and mounted in glycerine jelly using the acetolysis method of Erdtman (1960). The equatorial diameter and polar diameter of pollen grains were measured using a calibrated eyepiece micrometer. The ratio between polar diameter and equatorial diameter (P/E) was determined.

For SEM studies, the material was fixed in glutaraldehyde and osmium tetroxide, then enclosed in dialysis tubing and dehydrated in a graded ethanol series for treatment in a critical point drier (CPD). The dried material was then mounted on standard specimen stubs covered with "electrodag 915" or "silverdag" and coated with gold in a diode sputter coater for 5 minutes, and electron-micrographs were taken using a Philips SEM 515.

For TEM studies, the anther of *A. carambola* clone B17 and upper-whorl anthers of *A. bilimbi* were dissected in a drop of 0.1 M sodium cacodylate buffer at pH 6.8, containing 2% sucrose. A few drops of the molten 2% Difco Bacto agar (at 60°C) were added and mixed well. The agar containing the pollen grains was left to solidify and then cut into small segments (approximately 2 x 2 x 1 mm) to ensure uniform fixation. These segments

were fixed immediately for 2 hours at 4°C in a 3% solution of glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 6.8, containing 2% sucrose. The sample was subsequently rinsed with buffer containing decreasingly smaller amounts of sucrose, distilled water and finally post-fixed in 1% aqueous osmium tetroxide solution for 90 minutes at room temperature. The material was rinsed in distilled water and dehydrated in a graded ethanol series, followed by two changes in 100% acetone, all at room temperature. After dehydration, the material was transferred through acetone and epoxy resin mixtures—a mixture of Araldite CY212, DDSA (dodecenyl succinic anhydrate) and BDMA (benzil dimethylamine)—through a graded series and rotated on a "Rota-Torque" rotating apparatus. The samples were finally placed in pure firm epoxy resin and embedded material was left at room temperature for 1 hour and polymerized in an oven (60°C) for 4 days. Ultrathin sections in the pale gold range were made on a Reichert Jung Ultracut microtome using a glass knife. Sections were collected on plastic-carbon-coated copper grids and stained with 8% uranyl acetate (5 minutes) and lead citrate (10 minutes). The sections were examined and micrographs were taken on a Philip CM12 TEM operated at 80 kV.

Results

LM & SEM studies (Figs. 1 & 2; Table 1)

The pollen grains of both *Averrhoa* species are small to medium-sized and tricolpate. They are oblate spheroidal to subprolate. In both clones of *A. carambola* and *A. bilimbi*, the polar diameter ranged from 19.4 μm (*A. carambola* clone B2) to 30.1 μm (upper-whorl anthers of *A. bilimbi*), while the equatorial diameter ranged from 18.0 μm. *carambola* clone B2) to 27.3 μm (upper-whorl anthers of *A. bilimbi*). Pollen grains from the thrum-eyed form (clone B17) of *A. carambola* are significantly larger than those of the pin-eyed form (clone B2). Upper-whorl anthers of *A. bilimbi* produced significantly larger pollen grains than those from the lower whorl.

The colpus is long, nearly reaching the poles and equally spaced in the equatorial axis. The colpus is spindle-shaped and not uniform in width, i.e., broadening at the centre and tapering towards the poles. The colpus membrane is smooth and the size of the tectal perforations diminishes near the colpus.

The pollen wall or sporoderm consists of two layers, the exine and the intine, the exine being thicker. The tectum ornamentation is tectate-perforate (in *A. carambola*) or semitectate (in *A. bilimbi*) and its surface foveolate (in *A. carambola*) or reticulate (in *A. bilimbi*). There are no differences in pollen exine sculpturing between long- and short-styled flowers of *A. carambola*.

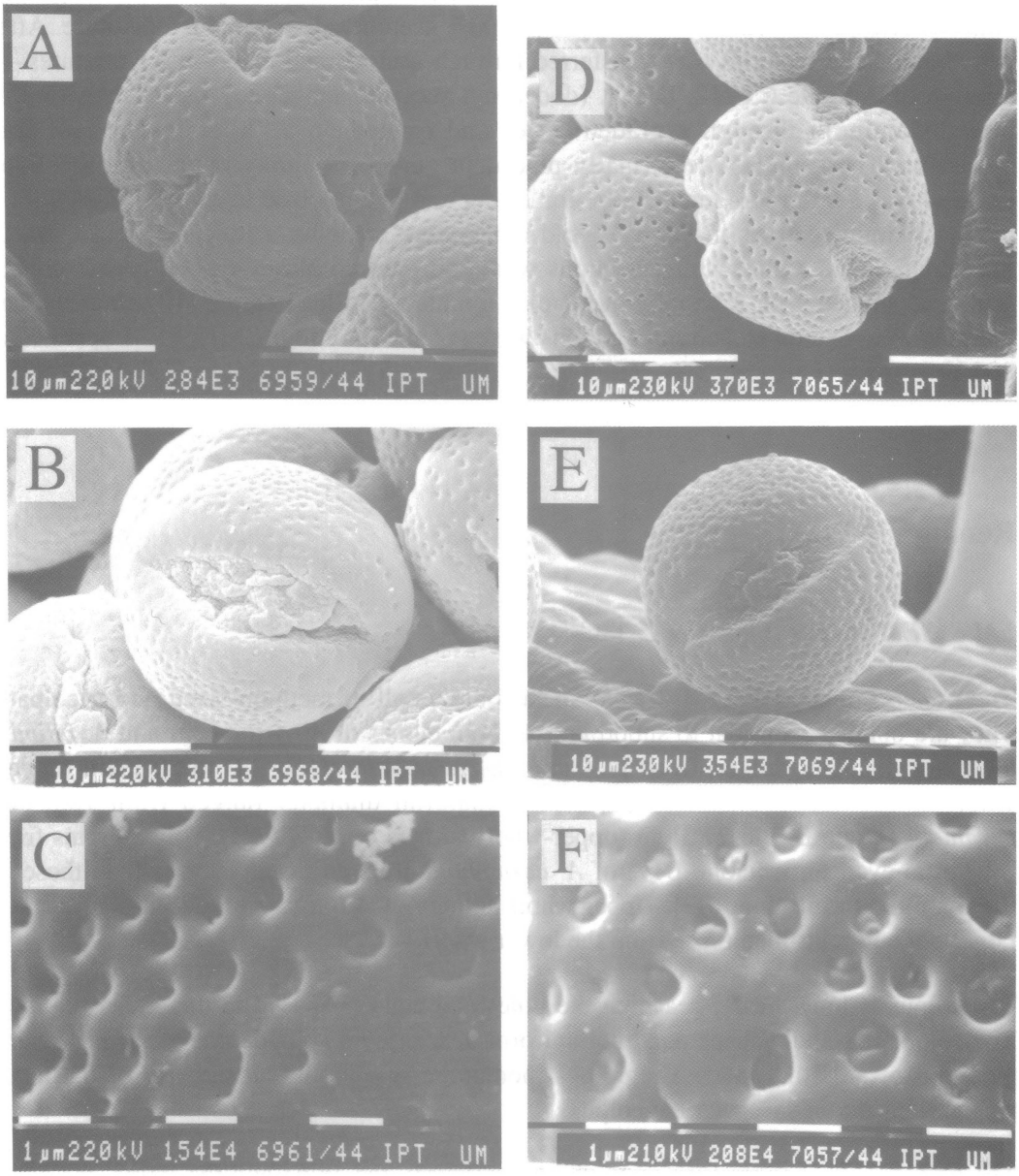

Fig. 1. SEM studies on *Averrhoa carambola* pollen. **A**, clone B17, polar view. **B,** clone B17, equatorial view. **C,** clone B17, pollen surface. **D,** clone B2, polar view. **E,** clone B2, equatorial view. **F,** clone B2, pollen surface. (Scale bars = 1 µm in C, F; 10 µm in A, B, D, E).

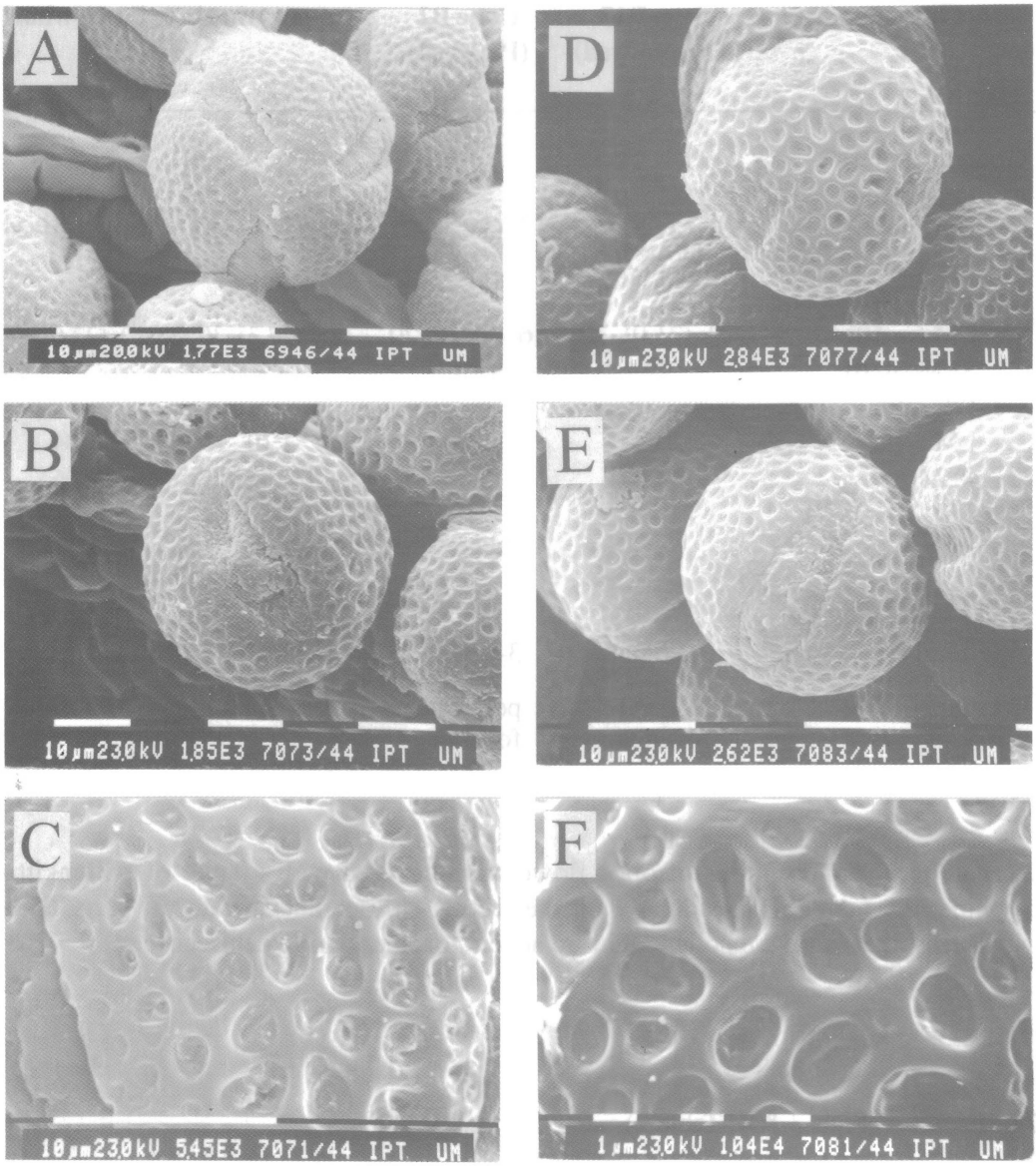

Fig. 2. SEM studies on *Averrhoa bilimbi* pollen. **A,** Pollen from upper-whorl anther, polar view. **B,** Pollen from upper-whorl anther, equatorial view. **C,** Pollen from upper-whorl anther, pollen surface. **D,** Pollen from lower-whorl anther, polar view. **E**, Pollen from lower-whorl anther, equatorial view. **F,** Pollen from lower-whorl anther, pollen surface. (Scale bars = 10 µm in Plates A-E; 1 µm in Plate F).

Table 1. Palynological characters of *A. carambola* and *A. bilimbi*, studied with LM and SEM.

Pollen Characters	*A. carambola* clone B17 (Thrum-eyed)	*A. carambola* clone B2 (Pin-eyed)	*A. bilimbi* upper whorl	*A. bilimbi* lower whorl
Grain size (μm)				
a. Polar axis:				
LM (n=50)	20–(21.8)–25	17–(19.4)–21	27–(30.1)–32	21–(23.5)–26
SEM (n=6)	23	19	33	22
b. Equatorial axis:				
LM (n=50)	19–(21.0)–22	16–(18.0)–20	25–(27.3)–30	20–(22.6)–24
SEM (n=6)	22	18	32	21
c. P/E ratio				
LM	1.04	1.08	1.10	1.04
SEM	1.05	1.06	1.05	1.06
Shape		oblate spheroidal – subprolate		
Colpus		nearly reaching poles		
Apertural type/system	3–colpate	3–colpate	3-colpate	3-colpate
Tectum	perforate foveolate	perforate foveolate	reticulate	reticulate

Unacetolysed pollen grains of open flowers are covered with a layer of pollenkitt or tryphine-like material, particularly on the tectum surface and infratectal parts. These substances may cause the pollen grains to adhere together during dehiscence.

TEM studies (Figs. 3 & 4; Table 2)

The exine in *Averrhoa* is characteristic of other dicotyledons in its basic organization. It is stratified, consisting of tectum, columellae, foot-layer and endexine. Exine thickness in *A. carambola* ranged from 0.9 to 2.0 μm (nexine 2/5 to 6/17 of sexine) whereas in *A. bilimbi,* it ranged from 1.5 to 2.4 μm (nexine 5/23 to 2/7 of sexine).

The tectum is characteristically well-developed, forming up to nearly a third of the total exine thickness. The tectum of *A. carambola* is continuous. The large gaps in the mesocolpial tectum are a reflection of the larger lumina in that area. However, the tectum is discontinuous in *A. bilimbi*, and is thicker than that of *A. carambola.*

The columellae in *A. carambola* are a prominent component and are not uniform in size, i.e., the columellae become shorter nearer the colpus. In contrast, those of *A. bilimbi* pollen are reduced, thin, of lesser extent and can be recognized as discrete entities. Both the foot-layer and endexine are more variable and do not react well to differential electron staining, making their separation and characterization difficult. The foot-layer is irregular in both; and it is thick in *A. carambola*, but thin in *A. bilimbi.* The endexine is present in all the exine regions of *A. bilimbi.* The endexine of both species is thinner than the foot-layer. There are free standing elements (bacula) in the lumina on the surfaces of *A. bilimbi* pollen.

The fibrillar intine is two-layered based on the staining affinity and is up to 0.6 μm in thickness. The undulating intine increases in thickness towards the apertural intine (oncus). Both the endintine and exintine are mostly continuous with the apertural intine.

Fig. 3. TEM photomicrograph of pollen wall ultrastructure in *Averrhoa carambola* clone B17 (**c**, columellate infratectal layer; **e**, exine; **fl**, foot-layer; **i**, intine; **n**, nexine; **p**, pollenkitt/tryphine; **s**, sexine; **sg**, starch containing plastid in cytoplasm; **t**, tectum; scale bar = 1 μm).

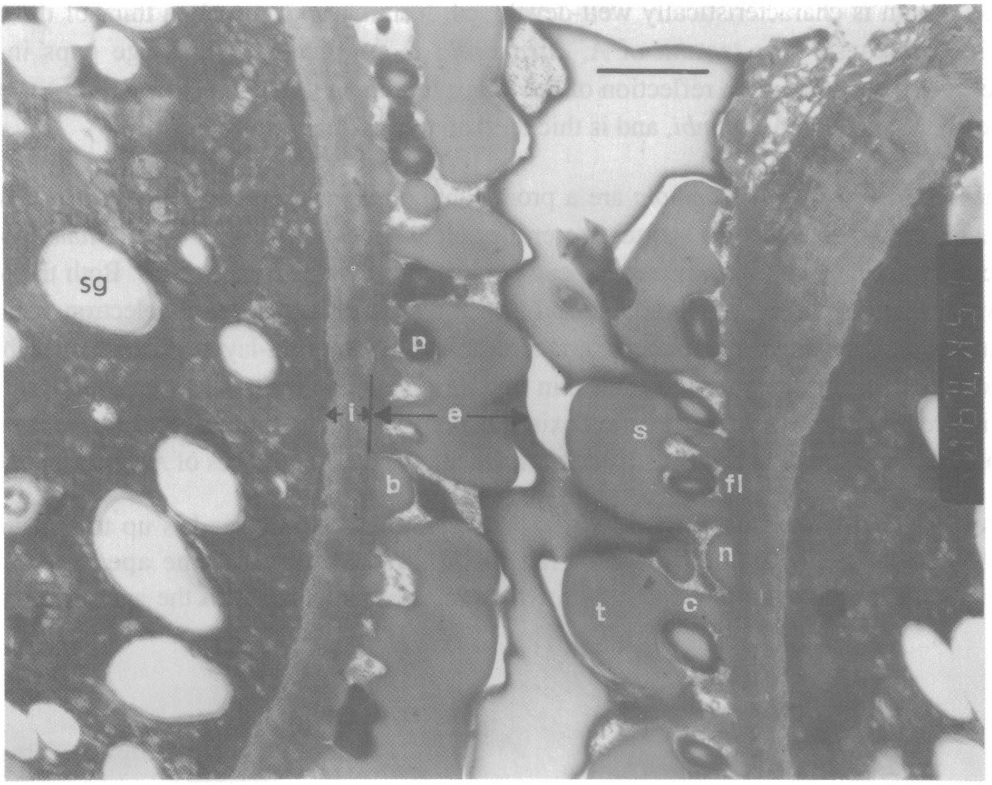

Fig. 4. TEM photomicrograph of pollen wall ultrastructure in *Averrhoa bilimbi* (**b**, bacula; **c**, columellate infratectal layer; **e**, exine; **fl**, foot-layer; **i**, intine; **n**, nexine; **p**, pollenkitt/tryphine; **s**, sexine; **sg**, starch containing plastid in cytoplasm; **t**, tectum; scale bar = 1 μm).

The cytoplasm of the mature pollen grain is occupied by many plastids containing starch. Endoplasmic reticulum and many mitochondria can also be seen in the cytoplasm.

Discussion

LM & SEM studies

As in other Oxalidaceae, the pollen grains in *Averrhoa* vary from oblate spheroidal, subprolate to prolate and from small to medium-sized spores (Erdtman 1966).

Table 2. Palynological characters of *A. carambola* and *A. bilimbi*, studied with TEM.

Pollen Characters	*A. carambola* clone B17	*A. bilimbi* upper whorl
Sexine	tectate	semitectate
Tectum	thin	thick
Infratectal layer	columellae	columellae
Columellae	uniform, prominent	thin discrete entities
Foot layer	thick	thin
Exine thickness (μm) (n=10)	0.9–2.0	1.5–2.4
Sexine thickness (μm) (n=10)	0.5–1.7	1.4–2.1
Nexine thickness (μm) (n=10)	0.2–0.6	0.3–0.6
Intine thickness (μm) (n=10)	0.3–0.6	0.3–0.6

Known polar diameter measurements of the pollen of other Oxalidaceae range from 21 μm (in *Biophytum abyssinicum*) to 63 μm (in *Oxalis bowicana*) (Erdtman 1966). 3-colpate pollen grains appear typical of *Averrhoa*, as has been reported for *A. carambola* (Ou Yang 1978), *A. bilimbi* (Tan 1977) and also *Oxalis acetosella* (Erdtman 1966). However, 3-colporoidate pollen grains have been observed in *Sarcotheca acuminata* and *Lepidobotrys staudtii*, and 3-colporate pollen grains have been found in *Hypseocharis pimpinellifolia* (Erdtman 1966).

The occurrence of two pollen sizes in *A. bilimbi* and in the two clones of *A. carambola* has not been recorded before the 1970s, when the studies of Tan (1977) and Ou Yang (1978) were made. The pollen size differences of heterostylous flowers also known from *Waltheria* (Sterculiaceae) (Köhler, 1976). According to Knuth (1906), pollen size differences occured in heterostylous flowers and each pollen size had its own function. Ganders (1979) found no relationship between pollen size and style length in 24 heteromorphic species. This strongly indicates that it is not necessary for thrum pollen grains to be larger in order to grow successfully down long pin styles. It is however,

possible that the asymmetry of pollen flow in most heterostylous plants (less pin pollen going to thrum stigmas) has been selected for the production of more and thus smaller pin grains, leading to a dimorphy in grain size and hence in pollen reserves (Ganders 1979).

Scanning electron microscope photomicrographs showed no differences in pollen exine sculpturing between long- and short-styled flowers of *A. carambola* and *A. bilimbi*, as has also been shown for boraginaceous genera (Ray & Chisaki 1957; Opler, Baker & Frankie 1975).

Chin & Phoon (1982) reported that under SEM the pollen grain of *A. carambola* was sticky and barrel-shaped at anther dehiscence. These pollen grains swelled up to about 20–24 μm when wet and revealed three large colpi. The exine is pockmarked with tiny spherical depressions. However, the present study shows that the pollen grain of *A. carambola* is oblate spheroidal to subprolate, measuring 18–23 μm and is perforate-foveolate. These differences may be a reflection of the preparation techniques.

TEM studies

As early as 1934, Potonié had recognized the difference between pollen structure and sculpturing and noted that this distinction was "absolutely necessary for the description of certain specimens". This distinction was also noted by Faegri & Iversen (1950, 1964). However, many studies, including that of Erdtman (1966), have failed to distinguish between structure and sculpturing when providing pollen grain descriptions. However, the scanning electron microscope will distinguish between pseudoreticulate and eureticulate pollen, while pollen wall sections, when observed with the transmission electron microscope, prove particularly helpful in distinguishing per-reticulate pollen from supra-reticulate pollen.

Averrhoa pollen exine is especially interesting because of its unique ultrastructural organization. The present study clarifies the fine structure of three principal sporoderm features, including the nature of the tectum and the structure of the infratectum and the nexine layers.

The pollen of heterostylous cordias (Boraginaceae) appear only weakly dimorphic in SEM or TEM (Nowicke, Miller & Bittner 1988). In heterostyly, pollen from the short-styled morph is generally larger than that of the long-styled morph, and this also appears to be the case in the present study. In sect. *Cordia* pollen from the short-styled morph is usually larger than that of the long-styled morph, but there is much variation within a sample (Nowicke & Miller 1990).

The germination and development of pollen tubes require energy, as well as the elaboration of wall-materials, callose, etc., within the tube, and these must be provided initially by reserves in the pollen grain, although some additional reserves may be picked up from stylar tissue. Such reserves may be in the form of fixed oils, sugars or starch (Ou Yang 1978, Baker & Baker 1979, Shivanna & Johri 1985). In the present study, the cytoplasm of mature *Averrhoa* pollen was seen to have many plastids containing starch. Baker & Baker (1979) have noted ten families, including the Oxalidaceae, that are characterized by starchy mature pollen grains. In insect-pollinated flowers where nectar is the only reward for the pollinator, there may be selection for starch-containing pollen. This occurs because metabolically, it will be easy to turn any sugars supplied to the developing pollen grains by the tapetum into starch rather than into oil, and also easy to break down starch into the sugars used in respiration, wall-building, and callose-production in the pollen tube (Baker & Baker 1979)

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